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(54) Title
RECOMBINANT VACCINIA VIRUS CAPABLE OF MULTIPLYING IN CHO CELLS

(57) Claim

However, there are some exceptions to this rule; in particular, the wild type of vaccinia virus is incapable of multiplying in a Chinese hamster ovary, CHO, cell line (Drillien, Spehner and Kirn, 1978). Thus, CHO cells constitute one of the most promising systems for the synthesis of proteins in mammalian cells. In fact, these cells can be cultured easily; they have a short generation time and their genetics are the best known of all similar systems.

The present invention relates to the modification of the vaccinia virus by integrating into its genome a foreign gene which gives it the capacity to multiply in CHO cells. The gene which provides this new host specificity is derived from the cowpox virus (or bovine smallpox, cow virus related to the vaccinia virus) which is capable of multiplying in CHO cells. The genomes of cowpox and of vaccinia virus are very similar particularly in the 100,000 base pairs of the central portion of DNA (Mackett and Archard, 1979). However, the genome of cowpox is larger than that of vaccinia (approximately 230,000 bp instead of 190,000) and the additional genetic information it contains, appears to reside essentially at

its ends. The similarity between the genome of vaccinia virus and that of cowpox enables the use of cowpox as the vector naturally adapted to CHO cells to be envisaged.

However, cowpox multiplies at a titer ten times lower than that of vaccinia, which is likely to result in a lower yield of expression of a protein produced by a cowpox recombinant in comparison with a vaccinia recombinant.

Moreover, from the point of view of using viral recombinants for vaccination, it must be emphasized that the use of vaccinia virus is well known and has now enabled smallpox to be eradicated completely.

The present invention relates to the development of a vector which has the known advantages of the vaccinia virus and the capacity of cowpox virus to multiply on CHO cells.

1. DNA sequence isolated especially from cowpox virus and which participates in the multiplication of this virus in CHO cells, and which contains all or a functional portion of the sequence shown in Figure 6 or a function equivalent sequence.

7. Plasmid vector containing a DNA sequence as claimed in one of claims 1 to 6.

8. Recombinant vaccinia virus containing a DNA sequence as claimed in one of claims 1 to 6.

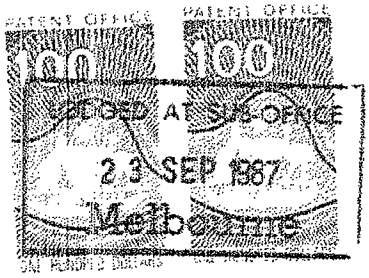
9. CHO cells infected with a recombinant vaccinia virus as claimed in claim 8.

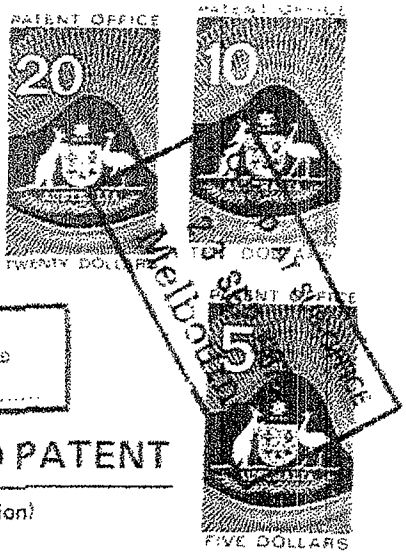
10. Method for the preparation of a protein of industrial value, wherein CHO cells as claimed in claim 9, infected with a recombinant vaccinia virus containing a gene coding for a protein of industrial value are cultured, the said gene being under the control of elements which provide for its expression in CHO cells.

Form 1

COMMONWEALTH OF AUSTRALIA

Patents Act 1952





FEE STAMP TO VALUE OF $ 235 ATTACHED
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APPLICATION FOR A STANDARD PATENT

(Combined Form – Convention and Non–Convention)

I/We ...TRANSGENE S.A., a French Body Corporate, of 16 rue Henri Regnault 92400 COUREVOIE, FRANCE,

hereby apply for the grant of a Standard Patent for an invention entitled DNA sequence, vectors, recombinant viruses and method which employs recombinant vaccinia viruses capable of multiplying in dog cells

which is described in the accompanying ~~Provisional~~ Complete Specification.

2. This application is a convention application and is based on the application(s) for a patent or similar protection made –

Strike out para.2. for non-convention

in France

on 23rd September, 1986, numbered 86 13272, and

on ....., numbered ....., and

on ....., numbered .....

3. My/Our address for service is: Care of COWIE, THOMSON & CARTER, Patent Attorneys, of 71 Queens Road, Melbourne, Victoria 3004, Australia.

DATED this 21st day of September, 1987.

To The Commissioner of Patents
~~COMMONWEALTH OF AUSTRALIA~~

LODGED AT SUB-OFFICE
23 SEP 1987
Melbourne

COWIE, THOMSON & CARTER
Patent Attorneys
71 Queens Road, Melbourne,
Victoria, 3004, Australia

COWIE, THOMSON & CARTER
By:

Patent Attorneys for
TRANSGENE S.A.

FORM 7 & 8

AUSTRALIA
PATENTS ACT 1952

PATENT DECLARATION FORM
(CONVENTION OR NON-CONVENTION)

DECLARATION IN SUPPORT OF APPLICATION FOR A PATENT

Insert name of applicant.

In support of the application made by [illegible]

Insert title of invention.

for a patent for an invention entitled [illegible] AND IN CHO CELLS

Insert full name(s) and address(es) of person(s) making declaration. If applicant a company person must be authorised to make declaration.

I/We c/o TRANSGENE [illegible]
C/O TRANSGENE S.A. 10 rue Henri Reinault
67100 COURSEVOIE - FRANCE

do solemnly and sincerely declare as follows:

* 1. (a) ~~I am/We are the applicant(s) for the patent.~~

* Delete alternatives which do not apply

* OR (b) I am authorized by the abovementioned applicant to make this declaration on its behalf.

* 2. (a) ~~I am/We are the actual inventor(s) of the invention.~~

Insert name(s) and address(es) of actual inventor(s).

* OR (b) L. Robert [illegible]
10 Boulevard Paul Deroulede, 67000 STRASBOURG
FRANCE

2. [illegible] CERTAIN
29 rue de la Chenaie, 67200 ROSHOLSHEIM
FRANCE

~~is~~/are the actual inventor(s) of the invention and the facts upon which the applicant(s) ~~is~~/~~are~~ entitled to make the application are as follows:—

Insert details of entitlement to apply, e.g. Applicant is assignee of inventor(s)

THE SAID COMPANY IS THE ASSIGNEE OF THE INVENTION FROM THE SAID ACTUAL INVENTORS

3. The basic application(s) as defined by Section 141 of the Act was/~~were~~ made in the following country or countries on the following date(s) by the following applicant(s)

Delete 3 and 4 if application non-convention. Otherwise insert details of basic application(s).

in FRANCE on 88 13272 September 29 19 88
by The applicant, TRANSGENE S.A.
in _____ on _____ 19 _____
by _____
in _____ on _____ 19 _____
by _____
in _____ on _____ 19 _____
by _____

4. The basic application~~(s)~~ referred to in paragraph 3 of this Declaration was/~~were~~ the first application(s) made in a Convention country in respect of the invention the subject of the application.

Place and date of Signature.

Declared at PARIS this 14 day of SEPTEMBER 19 87

NO ATTESTATION
OR SEAL

[signature]

Etienne [illegible] (Manager)
Signature(s) of declarant(s).

To: The Commissioner of Patents,
Australia

P/00/011

Form 10

PATENTS ACT 1952-1973

COMPLETE SPECIFICATION

(ORIGINAL)

FOR OFFICE USE

Class:

Int. Cl:

Application Number:
Lodged:

Complete Specification—Lodged:
Accepted:
Published:

Priority:

Related Art:

TO BE COMPLETED BY APPLICANT

Name of Applicant: TRANSGENE S.A., a French Body Corporate, of 16 rue Henri Regnault 92400 COUREVOIE, FRANCE.

Address of Applicant:

Actual Inventor: Robert Drillien & Danièle Spehner

Address for Service: COWIE, THOMSON & CARTER
PATENT & TRADEMARK ATTORNEYS
71 QUEENS ROAD
MELBOURNE, 3004, AUSTRALIA

Complete Specification for the invention entitled: DNA SEQUENCE VECTORS, RECOMBINANT VIRUSES AND METHOD WHICH EMPLOYS RECOMBINANT VACCINIA VIRUSES CAPABLE OF MULTIPLYING IN CHO CELLS

The following statement is a full description of this invention, including the best method of performing it known to me:—*

*Note: The description is to be typed in double spacing, pica type face, in an area not exceeding 250 mm in depth and 160 mm in width, on tough white paper of good quality and it is to be inserted inside this form.

11710/76-L

C. J. Thompson, Commonwealth Government Printer, Canberra

Vaccinia virus has increasingly been used as expression vector in animal cells since methods specific to this system were developed (Panicali and Paoletti, 1982; Macket et al., 1982, Smith et al., 1983; Panicali et al.; Kieny et al., 1984). The construction of recombinant viruses of vaccinia virus containing genes which code for proteins of medical or veterinary interest is particularly sought. The synthesis of the foreign protein whose gene has been integrated into the genome of vaccinia virus can then be obtained in in vitro cell culture or after inoculating into a living organism, depending on the object pursued. One of the advantages of vaccinia virus as the vector is its capacity to multiply in a large number of different types of cells.

However, there are some exceptions to this rule; in particular, the wild type of vaccinia virus is incapable of multiplying in a Chinese hamster ovary, CHO, cell line (Drillien, Spehner and Kirn, 1978). Thus, CHO cells constitute one of the most promising systems for the synthesis of proteins in mammalian cells. In fact, these cells can be cultured easily; they have a short generation time and their genetics are the best known of all similar systems.

The present invention relates to the modification of the vaccinia virus by integrating into its genome a foreign gene which gives it the capacity to multiply in CHO cells. The gene which provides this new host specificity is derived from the cowpox virus (or bovine smallpox, cow virus related to the vaccinia virus) which is capable of multiplying in CHO cells. The genomes of cowpox and of vaccinia virus are very similar particularly in the 100,000 base pairs of the central portion of DNA (Mackett and Archard, 1979). However, the genome of cowpox is larger than that of vaccinia (approximately 230,000 bp instead of 190,000) and the additional genetic information it contains, appears to reside essentially at its ends. The similarity between the genome of vaccinia virus and that of cowpox enables the use of cowpox as the vector naturally adapted to CHO cells to be envisaged.

However, cowpox multiplies at a titer ten times lower than that of vaccinia, which is likely to result in a lower yield of expression of a protein produced by a cowpox recombinant in comparison with a vaccinia recombinant.

Moreover, from the point of view of using viral recombinants for vaccination, it must be emphasized that the use of vaccinia virus is well known and has now enabled smallpox to be eradicated completely.

The present invention relates to the development of a vector which has the known advantages of the vaccinia virus and the capacity of cowpox virus to multiply on CHO cells.

Firstly, the present invention relates to the identification and the localization of the genetic information which gives the cowpox virus the capacity to multiply in CHO cells.

In fact, the studies carried out have made it possible to identify a sequence involved in the multiplication of cowpox virus in CHO cells and which, when transferred into the vaccinia virus, ensures the multiplication of this virus in the CHO cells.

Thus, the invention relates to a DNA sequence isolated especially from cowpox virus and which participates in the multiplication of this virus in CHO cells and which comprises the whole or a functional portion of the sequence shown in Figure 6 or a functionally equivalent sequence.

It is possible that a portion of this gene, referred to as the "functional portion", is sufficient to provide for the multiplication. It is also possible that mutations or point variations do not modify the function; reference is then made to "functionally equivalent sequence".

Although it is preferred that this DNA sequence contains its own control signals which provide for its expression in CHO cells, this is not indispensable, and placing it under the control of elements having a different origin may be envisaged.

As described above, this DNA sequence is more

particularly intended for integration into the vaccinia virus in order to provide for its multiplication in CHO cells. This integration is accomplished by homologous recombination; it is therefore useful to ensure that the DNA sequence in question will contain at least one region homologous to a sequence of the vaccinia virus which could participate in this process of homologous recombination during the intracellular multiplication of the viruses.

In fact, it appears that in the cowpox virus the gene which enables multiplication to occur in CHO cells is surrounded by sequences homologous to the sequences of the vaccinia genome, which enables the preparation of a plasmid recombination vector to be simplified.

As already known in the case of the vaccinia virus, it is possible to insert into the DNA sequence which forms the subject of the invention a gene coding for a protein of industrial value, which is dependent on the control elements providing for its expression in the host cells. This technology has already been described, especially in the following patents: 84/06,499, 84/07,959 and 85/09,225 and can be employed possibly with some adaptations and advantages. In particular, in the preceding constructions, the selection of the recombinant viruses was carried out by inserting the gene to be expressed into the TK gene of vaccinia, which results in the recombinant virus being rendered TK^- and in enabling the selection to be carried out by the known method.

In the present case, it is not indispensable to carry out an insertion into the TK gene because, insofar as the gene coding for the protein of industrial value is linked to the gene providing for the multiplication in CHO cells, only the recombinant viruses can multiply on CHO cells, which allows a "natural" selection of the recombinant viruses to be carried out.

In the latter case, it is preferable that the DNA block containing the genes to be recombined is flanked by sequences homologous to the sequences of vaccinia virus in order that they remain linked during the recombination.

The present invention also relates to CHO cells

infected with a recombinant vaccinia virus incorporating a DNA sequence as described above and, in particular, a gene coding for a protein of industrial value and to the corresponding viruses.

The invention also relates to plasmid vectors incorporating a DNA sequence as described above, it being possible for these vectors to be employed in order to carry out the recombination in vivo.

The invention also relates to CHO cells which have been integrated into a DNA sequence according to the present invention and which are capable of ensuring the multiplication of the vaccinia virus in these cells.

Finally, the invention relates to the preparation of proteins of industrial value by culturing CHO cells which are infected by a recombinant virus according to the invention.

The examples below are intended for illustrating the other characteristics and advantages of the present invention.

Example 1 Identification of the region of the cowpox virus genome which permits multiplication in CHO cells.

Recombinants between the vaccinia virus and cowpox were selected after mixed infection of chick embryo cells with each of the viruses. Analysis of the DNA of the recombinants shows that the capacity to multiply in CHO cells is associated with the retention of the restriction sites at the left end of the cowpox genome.

Primary chick embryo cells, prepared from 11 to 12-day old embryonated eggs, are simultaneously infected with a temperature-sensitive mutant of the vaccinia virus, tsN7 (Drillien et al., 1982) and cowpox (Brighton strain) at a rate of 2 plaque-forming units (pfu) per cell. Concurrently, other cell lawns are infected with each of these viruses. After one hour of adsorption, the excess unadsorbed virus is removed and fresh medium is added to the cells.

The latter are incubated at 33°C for one to two days until the entire cell layer undergoes necrosis. The

infected cells are then frozen and thawed, and the virus resulting from the infection is titrated at 39.5°C on thick embryo cells, under a layer of medium containing 1% refined agar. After two days at 39.5°C, a larger number of plaques of the virus is formed on the cell lawns infected with the mixture of the two viruses than on the control lawns (neither the temperature-sensitive mutant of vaccinia virus nor the cowpox gives a significant number of plaques); the plaques which appear from the mixed infection may therefore correspond to recombinants between the cowpox and the vaccinia virus.

Plaques of potential recombinants are then taken again individually, and the virus they contain is amplified by multiplication on chick embryo cells. Their DNA is then purified, cut with restriction enzymes and then analyzed on agarose gel.

It can be concluded from the restriction profiles that each plaque indeed corresponds to a recombinant between the vaccinia virus DNA and the cowpox DNA. Using known restriction maps for related viruses (Mackett and Archard 1978, Drillien and Spehner 1983), it is possible to establish the origin of most of the fragments of the recombinants and to draw their restriction maps (Figure 1).

It is seen that the recombinants designated 4, 6, 14, 15 and 19 which are capable of multiplying in CHO cells have retained the characteristic sites of the left end of the cowpox genome. The other recombinants designated 2, 7, 11, 16 and 18 which are incapable of multiplying in CHO cells have only a part or none of these sites of the left end of the cowpox genome.

It follows from these results that the retention of the restriction sites of the left end of the cowpox genome is associated with the phenotype of multiplication on CHO cells.

Example 2 Isolation and analysis of the genome of the vaccinia virus recombinants, which has integrated a cowpox DNA fragment

In order to define more accurately the localization of useful genetic information, recombinants capable

of multiplying on CHO cells were selected after infection with the vaccinia virus and transfection with cowpox DNA fragments.

The restriction fragments useful for the analysis of the portion which is important, i.e. the left end of the two viruses, are shown in Figure 2; the events of recombination described in Example 1 may be expected to occur in this portion of the genome.

Primary cells of thick embryo are infected with the vaccinia virus mutant tsN7 (Drillien et al., 1982) at a rate of 0.1 pfu per cell and transfected with a mixture of intact DNA of the wild strain of the vaccinia virus (Copenhagen strain) and the cowpox DNA (Brighton strain) which has previously been digested with the enzyme HindIII. Controls for transfection without DNA or with the DNA of the vaccinia virus alone are prepared. After incubating for 48 hours at 39.5°C, the cells are frozen, thawed and the virus thus released is titrated on a monolayer of CHO cells which are then covered with the medium containing 1% agar.

Samples originating from cells transfected with the cowpox DNA give many lytic plaques on CHO cells whereas the control samples give none of them.

The plaques visible on CHO cells are taken again individually and the virus they contain is amplified on chick embryo cells. Their DNA is then extracted and analyzed by comparison with the DNA of the two related strains of vaccinia and cowpox. After digesting with the enzyme EcoRI, the DNA fragments are separated by electrophoresis on agarose gel and they are then transferred to a nitrocellulose filter and hybridized with the SalI-K fragment of the vaccinia virus, which is radioactively labelled with ^{32}P.

After washing the nitrocellulose in order to remove the non-specifically bound radioactivity, an autoradiograph is prepared. The autoradiograph (Figure 3) shows that the recombinants of the vaccinia virus which have integrated a cowpox fragment have lost the EcoRI-C fragment which is typical of the vaccinia virus and they

contain an EcoRI fragment which hybridizes with the radioactive SalI-K vaccinia fragment; this fragment is intermediate in size between the EcoRI-A fragment of the cowpox and the EcoRI-C fragment of the vaccinia virus.

This new cowpox-vaccinia hybrid EcoRI fragment which is present in all the recombinants, originates from a double recombination between the EcoRI-A fragment of cowpox and the EcoRI-C fragment of vaccinia and must contain the information required for multiplication in CHO cells. For this recombination to take place, it was essential for the information which permits multiplication on CHO cells to be surrounded on either side by sequences of the cowpox genome homologous to the sequences of the vaccinia genome.

Example 3 Construction of a recombinant plasmid carrying the region of the cowpox genome, which permits multiplication in CHO cells.

In order to isolate the genetic information which permits multiplication in CHO cells, the EcoRI-A fragment of one of the recombinants described in Example 2 was cloned in the bacterial plasmid pAT153 (Twigg and Sherratt, 1980).

The DNA of one of the recombinants described in Example 2 is purified and then cut with the enzyme EcoRI. The EcoRI-A fragment is eluted from agarose gel and then inserted into the plasmid pAT153 which has previously been subjected to the action of EcoRI. HB101 bacteria are transformed with the ligation mixture and the DNA of the colonies obtained is then transferred onto nitrocellulose and hybridized with the SalI-K fragment of the vaccinia virus. The colonies which are hybridization-positive are amplified and the plasmid DNA they contain is purified. Two plasmids were obtained: pEA1 and pEA2, which correspond to the insertion of the EcoRI-A fragment into the two opposite orientations in the vector pAT153.

In order to verify that these plasmids carry the genetic information which permits the multiplication of the virus on CHO cells, a recombination is induced between the insert of the plasmid DNA and a vaccinia virus: chick

embryo cells are infected with the temperature-sensitive mutant tsN7 of the vaccinia virus at a rate of 0.1 pfu per cell and then transfected with the DNA of a wild vaccinia virus and with the DNA of the plasmid pEA1 or pEA2. Controls without the DNA of the vaccinia virus or without plasmid are also prepared. After incubating for 48 hours at 39.5°C, the cells are frozen and then thawed and the virus resulting from the infection is titrated on CHO cells. Only the samples originating from the cells transfected with the vaccinia virus DNA and the plasmids pEA1 or pEA2 give plaques on CHO cells.

Example 4 Sub-cloning of pEA1 fragments of smaller size in a vector plasmid intended for recombination with the vaccinia virus.

In order to improve the localization of the genetic information which permits the multiplication in CHO cells, restriction fragments which cover smaller portions of the plasmid pEA1 were cloned in a plasmid carrying the thymidine kinase (TK) gene of the vaccinia virus: pTG186poly.

Construction of pTG186poly:

The HindIII fragment (Hin-J) of the genome of the vaccinia virus (VV) contains the complete thymidine kinase (TK) gene which has already been used previously to permit the exchange and the recombination of a foreign DNA fragment in the VV genome (Mackett et al., 1982).

It is important to note that the transfer of an insert into the TK gene of the VV genome produces a TK-deficient virus, which facilitates its selection.

It was first of all necessary to produce a plasmid of small size carrying a unique HindIII site which can be used for integrating the Hin-J fragment of VV. Additionally, the unnecessary restriction sites of the plasmid had to be removed so as to enable the subsequent operations to be carried out.

The construction was initiated starting with the plasmid pML2 (Lusky and Botchan, 1981) which is a vector derived from the plasmid pBR322 in which the segment between the nucleotides 1089 and 2491 was lost by spontaneous

deletion. The PstI sequence was first removed by inserting the AhaIII-AhaIII fragment of pUC8 (Vioira and Messing, 1982) between the two AhaIII sites of pML3, removing 19 base pairs.

The "linker-tailing" method (Lathe et al., 1984) was employed to insert a HindIII adapter between the NruI and EcoRI sites treated with S1 of this plasmid, removing the BamHI site. This leads to a plasmid with 2049 base pairs carrying the functional β-lactamase gene (which gives ampicillin resistance) and additionally containing an origin of replication which is active in E.coli and a unique HindIII restriction site. This construction was called pTG1H.

The Hin-J fragment of the DNA of VV carrying the TK gene has previously been cloned into the pAT153 vector (Drillien and Spehner, 1983). This 4.6-kb fragment was recloned into the HindIII site of pTG1H. A clone in which the TK gene is located distally relative to the gene coding for ampicillin resistance was selected. This construction was called pTG1H-TK.

The pTG1H-TK construction was used as a vector for the following constructions.

The next step was to isolate a promoter for VV, which can be used to control the expression of the foreign gene to be integrated into VV. The promoter for an early gene coding for a 7,500-dalton (7.5 K) protein has already been successfully used for an identical purpose (Smith et al., 1983), and an isolation of this segment was therefore carried out.

The 7.5 K gene is located on one of the smallest SalI fragments (Sal-S fragment) of the genome of the WR type of VV (Venkatasan et al., 1981). As the small fragments were cloned preferentially, a large proportion of the clones obtained by cloning the DNA of the WR type of VV cut with SalI directly into the pBR322 plasmid carries the Sal-S fragment. This fragment is transferred onto the vector bacteriophage M13mp701 (Kieny et al., 1983), by SalI digestion and religation, which gives the phage M13.TG.Sal-S.

In this clone, a ScaI site is present in the immediate proximity of the initiation ATG of the 7.5 K gene. Downstream of the 7.5 K gene there are two unique BamHI and EcoRI sites originating from the vector. The BamHI and ScaI sites are fused using a BglII adapter: 5'-CAGATCTG-3' after filling in the ends generated by BamHI digestion with the Klenow fragment of E. coli polymerase. This method removed the ScaI site, but reconstitutes the BamHI site and moves the unique EcoRI site downstream. At the same time, the SalI (AccI) site downstream is removed, and the SalI site upstream therefore becomes unique. This construction is called M13.TG.7.5K.

Within the Hin-J fragment of the DNA of VV there are ClaI and EcoRI sites which are separated by approximately 30 base pairs (Weir and Moss, 1983). The 7.5 K promoter fragment present in M13.TG.7.5K is excised with AccI and EcoRI and cloned between the ClaI and EcoRI sites of pTG1H-TK in order to produce pTG1H-TK-P7.5K.

In this construction, the unique BamHI and EcoRI sites of the M13 vector are located immediately downstream of the 7.5 K promoter sequence. These unique BamHI and EcoRI sites are used in the following construction.

The polylinker segment of the bacteriophage M13TG131 (Kieny et al., 1983) is excised with EcoRI and BglII and inserted between the EcoRI and BamHI sites of the plasmid pTG1H-TK-P7.5K, generating pTG186poly. In this construction, 5 unique restriction sites are available for cloning a foreign gene under the control of the P7.5K promoter: PstI, BamHI, SstI, SmaI and EcoRI.

Insertion of the pEA1 fragments into pTG186poly:

The EcoRIA fragment of the plasmid pEA1 was digested with different enzymes, and fragments of different sizes were inserted into pTG186poly.

The recombinant plasmids derived from pTG186poly and containing portions of the EcoRI-A fragment of the recombinant plasmid pEA1 are shown in Figure 4.

Cutting of the plasmid pEA1 with the enzyme BglII gives several fragments. The largest of the BglII fragments was inserted into the BamHI site of the